

GENETIC MARKERS FOR IDENTIFICATION OF SOUTHERN PINE SPECIES

Rabiu Olatinwo, D. Paul Jackson, Shi-Jean S. Sung, Alex Mangini, Brian Strom, and James P. Barnett

Abstract—The chloroplast DNA (cpDNA) is paternally inherited in pine species (*Pinus* spp.), hence potentially useful in developing genetic markers for four common southern pine species [longleaf pine (*P. palustris* Mill.), loblolly pine (*P. taeda* L.), shortleaf pine (*P. echinata* Mill.), and slash pine (*P. elliottii* Engelm.)]. In this study, we (a) developed a simple DNA tool to accurately distinguish pure longleaf pine seeds or seedlings from other pine species, a critically important aspect of any longleaf pine restoration effort, and (b) validated the accuracy of identified genetic marker/specific primer combinations that uniquely identify the four pine species. Four genetic markers identified in this study correctly distinguished the four southern pine species based on the evaluation of over 200 tissue samples from multiple sources in Arkansas, Louisiana, and Mississippi that were tested. This DNA-based tool will enable routine and timely detection of natural hybridization when screening for seeds or seedlings intended for the restoration of pine ecosystems in southern region of the United States.

INTRODUCTION

The longleaf pine (*Pinus palustris* Mill.) restoration efforts (Guldin and others 2016) in the Southern Region have led to an increased demand for longleaf seeds from different sources, including seed orchards in Louisiana and Mississippi. Although most longleaf pine seeds collected from established orchards are pure longleaf, a small percentage are hybrids. Generally, a level of less than 3 percent of hybrids in any longleaf pine seedlot or seedling crop is considered acceptable. However, in recent years, there are indications that longleaf pine seedling lots have demonstrated less desirable traits (Barnett and others 2020), and have been presumed to be a naturally occurring hybrid between longleaf pine and loblolly pine (*Pinus taeda* L.) known as Sonderegger pine (*Pinus x sondereggeri* H.H.Chapm.) (Chapman 1922). For decades, such hybrids have been considered much less desirable than either of the parent species (longleaf or loblolly) because of their susceptibility to pests and extra-large branches (Barnett and others 2002, Wakeley 1954), thereby creating a need for an accurate detection of and timely determination between longleaf and Sonderegger pines. The issue of natural hybridization is a problem not restricted to longleaf, but found in other southern pine species, including shortleaf pine (*Pinus echinata* Mill.) hybridizing with loblolly pine (Stewart and others 2012).

How can managers accurately distinguish a pure longleaf from a Sonderegger hybrid or any of the other southern pine species? Although morphological determination may be possible between parent pine species and hybrids, it may be subjective and inconsistent (fig. 1). Can the true identity of southern pine seeds/seedlings/saplings/trees be determined using DNA techniques? The objective of the study was to develop a DNA-based tool to accurately distinguish “pure” longleaf pines (seeds or seedling/sapling/tree) from other southern pine species by using the chloroplast DNA (cpDNA), which is paternally inherited in pine species via pollen (Chen and others 2002, Neale and Sederoff 1989). Our objectives were to (a) identify genetic markers and develop a simple DNA tool to accurately distinguish a pure longleaf pine seed or seedling/sapling/tree from other southern pine species, and (b) validate the accuracy of selected sets of genetic marker/specific primer combinations that uniquely identify each of the four southern pine species. By achieving these objectives, markers identified in this study could serve as a critically important tool in detecting any natural hybridization within seed sources and accurately identifying pine species intended for restoration efforts.

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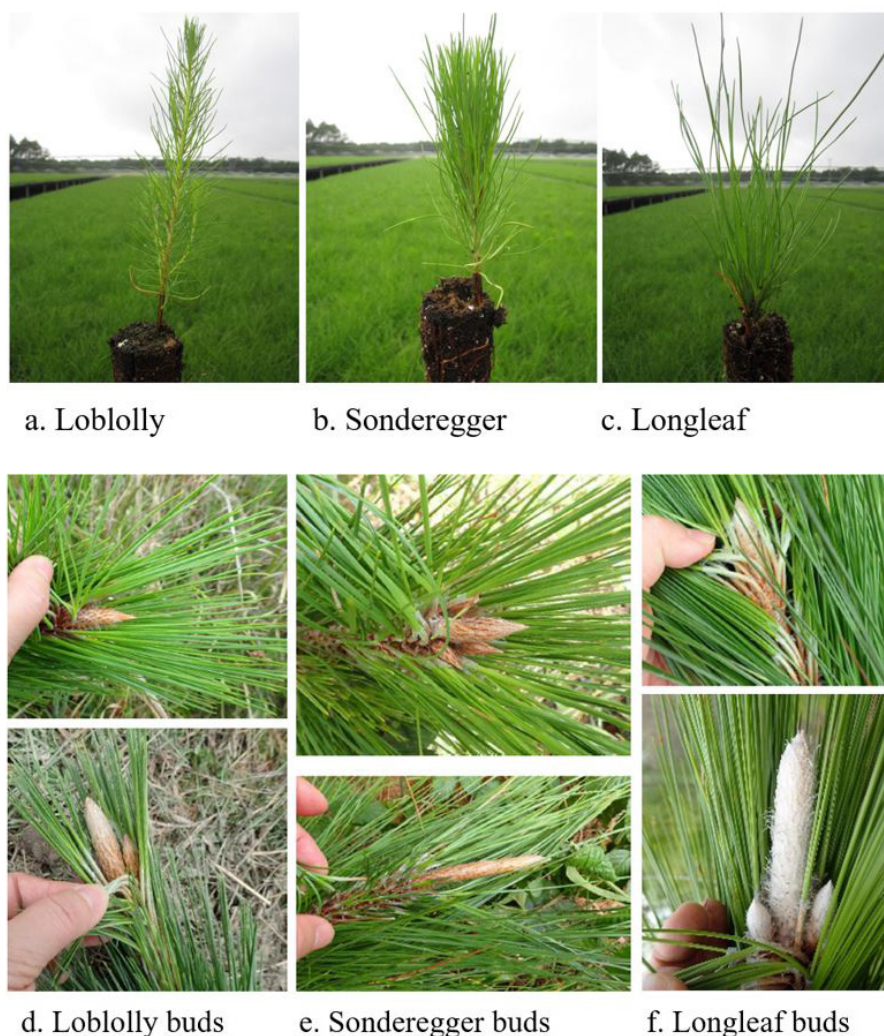


Figure 1—Stem elongation and bud morphology have been traditionally used to distinguish loblolly, longleaf, and their hybrid (Sonderregger) pine trees in the nursery (a-c) and field (d-f).

MATERIALS AND METHODS

Pine Tissue Samples

In this study, a total of 231 pine tissue samples obtained from multiple sources in Arkansas, Louisiana, and Mississippi were evaluated. A total of 64 needle samples were collected from 29 pine seedlings grown from stored longleaf pine seeds from the LA 2014 seedlot (U.S. Department of Agriculture, Forest Service, Stuart Seed Orchard, Bentley, LA), 17 seedlings from the MFC 2014 seedlot (Mississippi Forestry Commission Seed Orchard), and 18 seedlings from the MFC 2013 seedlot. Fifty needle samples were obtained from 25 mature loblolly and 25 longleaf pine trees at the Stuart Seed Orchard, while a similar set of 50 needle samples were obtained from trees on the Mississippi Forestry Commission Seed Orchard. Eighteen stem cambial tissue samples from different pine species located at the Crossett Experimental Forest in Crossett, AR, were included in the evaluation. Twenty-two needle samples

from seedlings grown from a mixed slash pine (*P. elliottii* Engelm.) seed sources from Louisiana were included in the genetic marker evaluations. Additional needle samples were obtained from a Sonderregger hybrid study plot (18 suspected hybrids and 3 longleaf saplings) established in 2010 and six saplings from another study established in 2010 on the Palustris Experimental Forest within the Kisatchie National Forest in Rapides Parish, LA. Measurements of total height (cm), ground-line diameter (mm), and diameter at breast height (mm) were made between 2011 and 2015 at the Sonderregger hybrid site. Data analysis was conducted in SAS-JMP v. 13.0 statistical software based on determination of the pine species or hybrid according to the chloroplast DNA markers.

Genetic Markers Identification

Variation in the complete chloroplast DNA sequences, specifically the single nucleotide polymorphism (SNP),

was explored and used for developing unique DNA markers for each of four southern pine species: longleaf pine, loblolly pine, shortleaf pine, and slash pine. The complete chloroplast genome sequences of pine species including the four southern pine species were obtained from the National Center for Biotechnology Information (NCBI) GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple aligned sequences were analyzed to obtain suitable fingerprints (DNA markers) for each of four southern pines (table 1). The GenBank accession for reference sequences for #1, #2, #3, and #4 markers were, respectively, JN854176 (longleaf pine), KC427273 (loblolly pine), JN854202 (slash pine), and JN854204 (shortleaf pine) (table 1). Forward and reverse polymerase chain reaction (PCR) primers were designed for each of the four markers to produce either a positive band or a null amplification (negative) on gel electrophoresis (table 2).

DNA Extraction

DNA was extracted from all pine tissue samples obtained for this study using the Qiagen DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The DNA from previously verified and known longleaf, loblolly, shortleaf, and slash pine tissue samples were included in the PCR amplifications as positive checks for the corresponding set of specific markers identified and evaluated in this study.

PCR Amplification

The PCR amplification of the DNA template was performed in a 10 µl PCR reaction in an Eppendorf Mastercycler® Pro PCR machine (Eppendorf AG Hamburg, Germany). The PCR was performed using the following conditions: 94 °C for 2 minutes → 94 °C

Table 1—Chloroplast DNA (cpDNA) markers identified for the detection of four southern pine species: longleaf pine, loblolly pine, shortleaf pine, and slash pine

cpDNA marker	Southern pine species	Reference seq. accession number	Single nucleotide polymorphism site	Start position
#1	Longleaf	JN854176	(TTCCGA) ²	119023
#2	Loblolly	KC427273	ATATATC*	96129
#3	Slash	JN854202	TACC	68085
#4	Shortleaf	JN854204	(CCATT) ²	42451

*Absent/missing in loblolly but present in the other southern pine species.

Table 2—Information on sets of specific PCR primers used for the detection of four southern pine species: longleaf pine, loblolly pine, shortleaf pine, and slash pine

Chloroplast DNA marker	Southern pine species	PCR sequence, 5' - 3'	Single nucleotide polymorphism target
#1	Longleaf	Forward: CTCATCATTTTCCGATTCCG Reverse: GGAAATGAAACACCGGAAGA	(TTCCGA) ²
#2	Loblolly	Forward: GTTCGAAAGAACAACAAGTAATATAG Reverse: CATAGCCAGGTTTTCCCAAA	ATATATC*
#3	Slash	Forward: GTCAACTAAAAAGAAGTAAAAATACC Reverse: CATTTTATTTCATAAGATAGATGCCAGA	TACC
#4	Shortleaf	Forward: AAATCATTTCCATTCCATTCAATT Reverse: TCGATAACGAATCCTATTTCATCC	(CATT) ²

*Absent/missing in loblolly but present in the other southern pine species.

PCR = Polymerase chain reaction.

for 30 seconds, 59 °C for 30 seconds, 72 °C for 1 minute, 30 cycles → 72 °C for 15 minutes, 4 °C hold, for amplification. The gel electrophoresis of 5 µl of each amplified PCR products was conducted on 1-percent agarose gel. The agarose was stained with ethidium bromide after 20 minutes of electrophoresis, and the resulting bands were visualized under ultraviolet (UV) illumination. Presence of a band on a gel indicates positive amplification, while no band indicates negative. A positive band with the longleaf marker identifies a sample as a longleaf pine, while a negative (no band) with longleaf marker indicates the sample is not a pure longleaf pine. Similarly, a positive band with any of the specific markers (i.e., #1, #2, #3, and #4) confirms the identity of the targeted pine species sample, while a negative or null band indicates the sample is not that pine species targeted. The accuracy of markers was validated with samples from different pine tissues obtained from multiple sources in Arkansas, Louisiana, and Mississippi.

RESULTS

Four species-specific DNA markers and specific primers developed to amplify these markers accurately detected the corresponding four southern pine species based on the chloroplast DNA from pine tissue samples

collected from multiple sources in Arkansas, Louisiana, and Mississippi. The genetic markers #1, #3, and #4 specifically detected longleaf, slash, and shortleaf pines, respectively; whereas marker #2 detected loblolly pine (paternal DNA) as shown in figures 2, 3, and 4. Of 18 cambial tissue samples from Arkansas, samples #1, 4, 5, 7, 8, and 10-12 were loblolly pine and the rest of the samples were shortleaf pine (fig. 2). The longleaf pine marker did not produce positive bands with these cambial tissue samples (fig. 2). The DNA classification results corroborated the tree species where the samples were obtained. The DNA classification results for 22 needle samples from seedlings of a mixed slash pine seedlot showed that samples #12, 14, 16, 17, and 20 were of loblolly pine, with the rest being slash pine except for #8 (fig. 3). Sample #8 was subsequently identified as slash pine when re-extracted DNA was tested for the four markers. Among 27 needle samples from the hybrid study plot in Louisiana, samples #13, 22-23, and 26-28 were identified as longleaf pine (fig. 4). The results from the 67 samples presented represent a cross section of the 231 samples evaluated.

Based on a longleaf marker (cpDNA Marker #1) classification of 21 pine saplings from the suspected “Sonderegger hybrid” plot, total heights of the

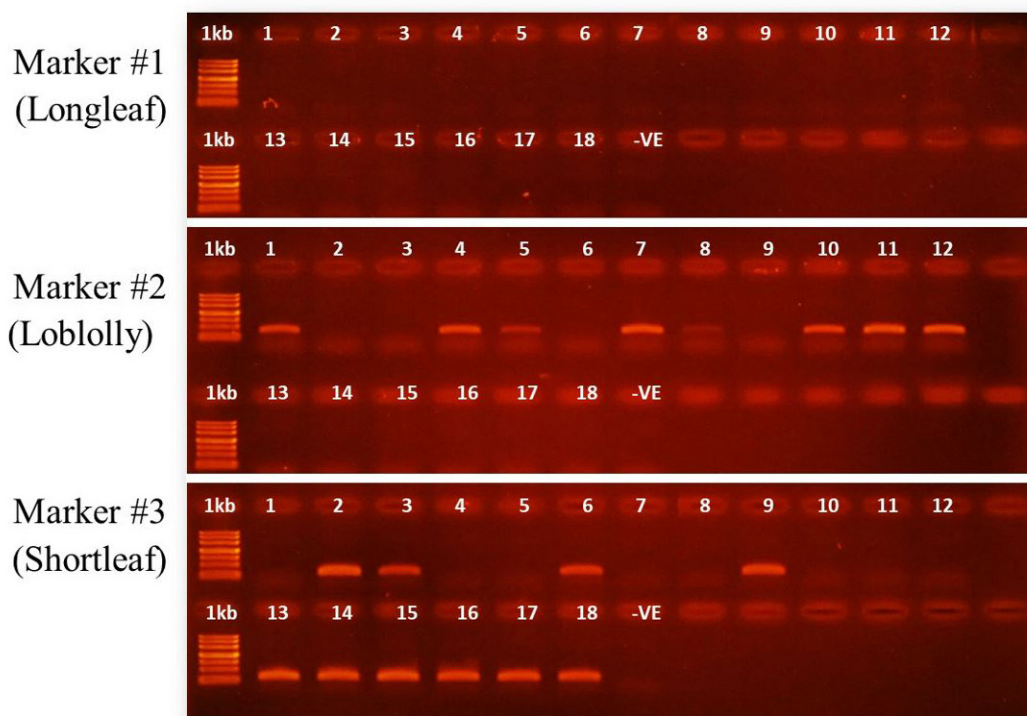


Figure 2—Detection of loblolly and shortleaf pines using three species-specific chloroplast DNA markers on DNA extracted from 18 pine stem cambium tissue samples from Crossett, AR.

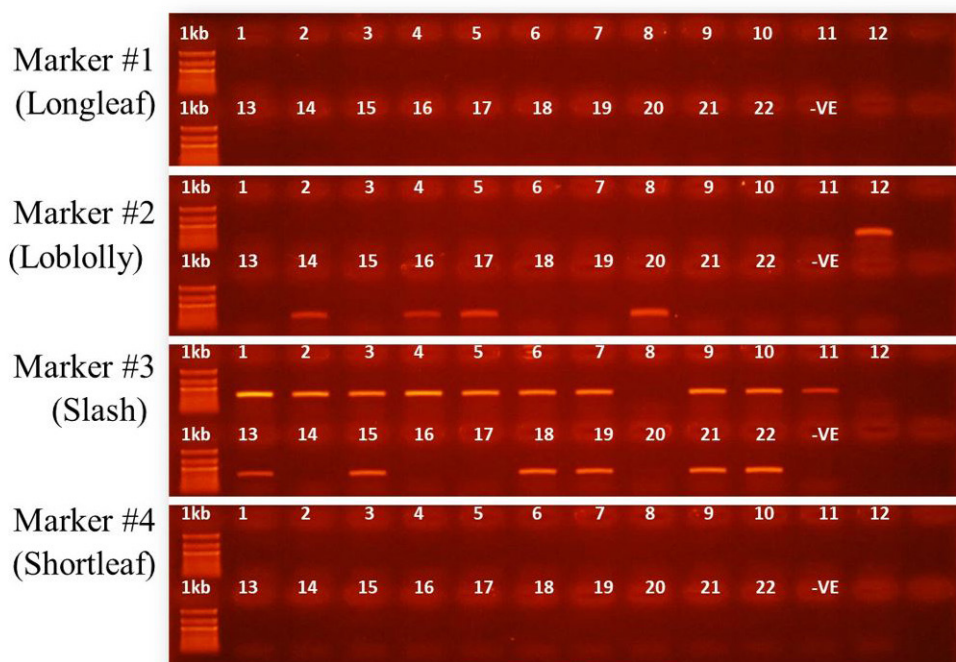


Figure 3—Detection of loblolly and slash pines using four species specific chloroplast DNA markers on DNA extracted from needle tissue samples of 22 seedlings from a mixed slash pine seedlot from Louisiana. Sample #8 was subsequently identified as slash pine when re-extracted DNA was tested for the four markers.

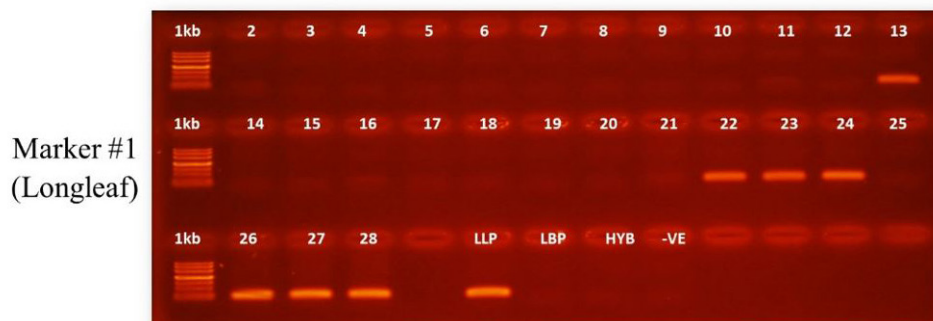


Figure 4—Identification of longleaf pine among 7- or 8-year-old Sonderegger hybrid saplings with a longleaf paternal marker (chloroplast DNA Marker #1) using DNA extracted from needle tissue samples collected from a Sonderegger plot located on the Palustris Experimental Forest within the Kisatchie National Forest, Rapides Parish, LA. Saplings were outplanted in 2009 (#24-#28) and 2010 (#2-#23). LLP, LBP, and HYB are the checks for longleaf, loblolly, and the Sonderegger hybrid, respectively.

verified Sonderegger hybrid were significantly greater compared to that of longleaf pine up to 5 years after planting (fig. 5). The initial mean seedling total height for Sonderegger pine and longleaf pine were 6.9 cm and 3.8 cm, respectively. After 5 years, Sonderegger pine and longleaf pine saplings had mean total height of 387.5 cm and 270.3 cm, respectively (fig. 5). After 3 years, average ground-line diameter was significantly larger for the Sonderegger hybrid than the longleaf pine with an average of 59.5 mm and 51.4 mm, respectively (fig. 6). However, at the end of the fifth year, the average diameter at breast height (d.b.h.) of the Sonderegger pine saplings was no longer statistically significantly greater than that of the longleaf pine saplings (fig. 6).

DISCUSSION

In this study, we identified species-specific cpDNA genetic markers (paternal) and developed a DNA-based tool to differentiate a “pure” longleaf pine (seed or seedling/sapling/tree) from other southern pine species by targeting paternally inherited cpDNA passed to progeny via pollen. The genetic markers/gel electrophoresis method described here explored the genetic variation and SNP target sites identified within the available cpDNA sequences. This technology addresses the emerging issues of natural hybridization in the southern pine forests.

Growth measurements made on seedling/saplings of the Sonderegger hybrid plot showed that this hybrid between longleaf and loblolly pine had greater growth in total height and stem diameter compared to that of the longleaf pine (figs. 5 and 6). Schmidtling (1999) observed the height growth in hybrid seedlings started almost immediately after germination, with the early growth much better than for longleaf pine but possessing the undesirable characteristics of loblolly pine such as poor form and susceptibility to fusiform rust disease. Hence, we concluded that a longleaf seedling that begins height growth in the nursery is likely a hybrid. We have yet to observe our Sonderegger saplings possess any undesirable form up to 9 years after planting.

Many landowners may decline to plant a Sonderegger hybrid seedling based on the observations by Chapman (1922) and Schmidtling (1999). In the nursery operation, longleaf seedlings with some degree of stem elongation are typically culled due to the poor quality of plants attributed to hybridization (Wakeley 1954). Generally, trees classified as Sonderegger pines are considered inferior to pure longleaf pine. Therefore, such an attribute may have serious consequences on marketability of longleaf pine seedlings at the nursery, and perhaps on the overall quality of seedlings available for use in the longleaf restoration initiative efforts. However,

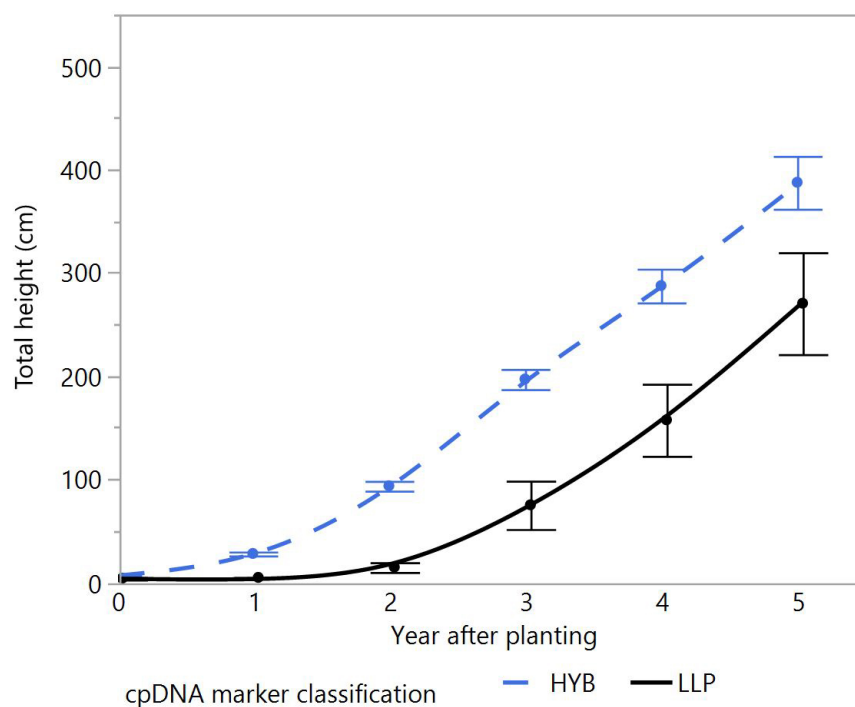


Figure 5—Total height of longleaf pine (LLP) and Sonderegger hybrid (HYB) saplings based on a longleaf marker (chloroplast DNA Marker #1) classification of 21 pine saplings from the Sonderegger hybrid plot on the Palustris Experimental Forest in the Kisatchie National Forest, Rapides Parish, LA.

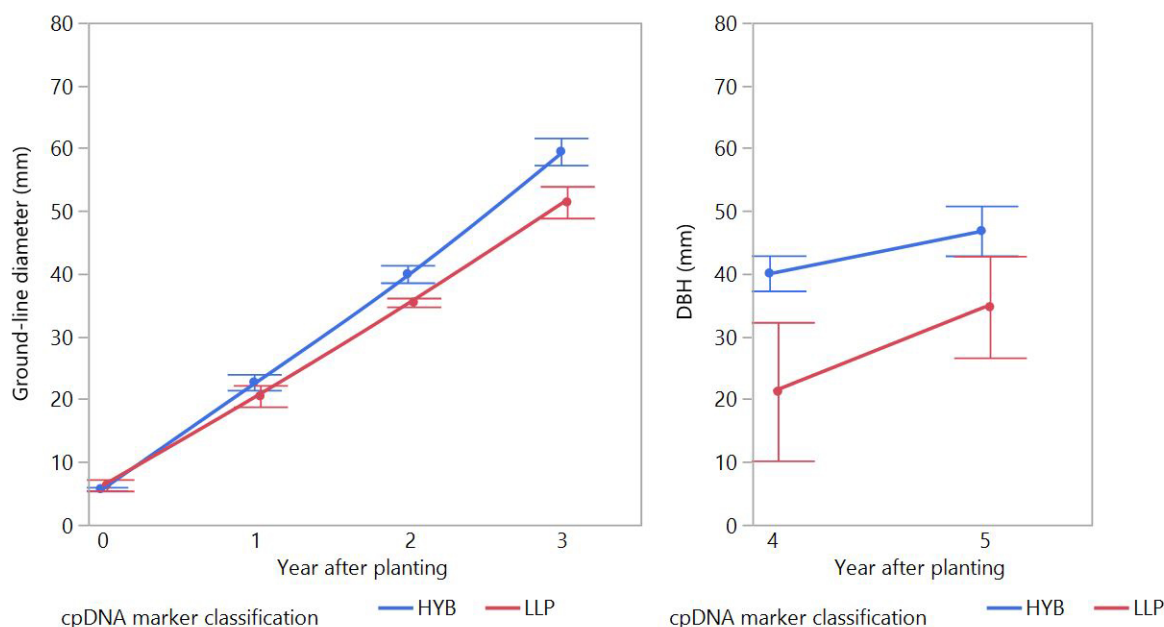


Figure 6—Ground-line diameter and diameter at breast height (d.b.h.) of longleaf pine (LLP) and Sonderegger hybrid (HYB) saplings based on longleaf marker (chloroplast DNA Marker #1) classification of 21 pine saplings from the Sonderegger hybrid plot on the Palustris Experimental Forest in the Kisatchie National Forest, Rapides Parish, LA.

measurements from trees evaluated at the Sonderegger hybrid plot at the Palustris Experimental Forest indicate that the average total height and stem diameter in Sonderegger hybrid pines were significantly greater than those of longleaf pines.

On the regional level, the U.S. Department of Agriculture, Forest Service, Southern Region initiated an ongoing effort of genotyping seed orchard clones and seedlots, which is an attempt to identify and eliminate the hybrid types from further contributing to the next generation of seed orchard seed crops and forest tree nursery seedling crops (Stewart and others 2016). Interestingly, one positive attribute of hybridization, at least in shortleaf pine × loblolly pine hybrids, is that they are more resistant to diseases compared to either parent. Shortleaf pine is susceptible to little leaf disease (caused by *Phytophthora cinnamomi*), and loblolly pine is susceptible to fusiform rust (caused by *Cronartium quercuum* f.sp. *fusiforme*) (Stewart and others 2016), while shortleaf x loblolly hybrids are less susceptible to these diseases (Benson and others 1982, Kraus 1986). Shortleaf pine x loblolly pine hybrids have also grown as well as or even better than one or both parents and have shown increased resistance to cold and ice damage (LaFarge and Kraus 1977).

Overall, identification of new DNA markers and improving the existing molecular tools will enable a better understanding of genetic diversity of southern

pin. Findings from this investigation offer a simple and relatively quick, but accurate, detection approach that will facilitate routine detection of “suspect longleaf pine,” namely Sonderegger hybrids, among pure longleaf pine seeds and seedlings. This method offers a way to estimate the extent of hybrid contamination in seedlots, in crops of pine seedlings at the nurseries, and in pine stands.

CONCLUSIONS

The genetic markers and the specific primers we developed accurately identified the four southern pine species evaluated in this study. We anticipate this DNA tool will facilitate routine detection of natural hybridization when screening for seeds or seedlings intended for the restoration of longleaf pine ecosystems in southern region of the United States.

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